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Cytokines driving breast cancer stemness

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ABSTRACT

There is increasing evidence for the cancer stem cell model in which a subset of cancer cells possessing stem cell properties, referred to as tumor-initiating or cancer stem-like cells (CSCs), play crucial roles in multiple aspects of cancer. Recent studies have started to characterize the crucial role of various cytokines in the tumor microenvironment in regulating the fate of CSCs. In this review, we summarized some of the latest findings on cytokines that drive breast cancer stemness and their mechanisms of action. These cytokines, including IL-6, IL-8, CCL2 and TGF-β, are frequently elevated in breast tumors and may hold promise as potential therapeutic targets to eradicate CSCs. In combination with conventional chemotherapy and radiotherapy targeting rapidly proliferating cancer cells, intervention of the cancer stemness-driving cytokines may achieve additional benefits for breast cancer patients by suppressing CSC-promoted cancer progression, recurrence, and drug refractoriness.

1. The CSC model in breast cancer

The CSC hypothesis is based upon the observations that cancer cells composing a tumor entity are phenotypically heterogeneous, and that only a subset of cancer cells can repopulate a new heterogeneous tumor. First demonstrated in acute myeloid leukemia in 1994 (Lapidot et al., 1994), this rare population of tumor-initiating cells are capable of sustaining tumor growth by generating differentiated cells with diverging phenotypes as well as undergoing self-renewal. As such, these cells stand at the top of a hierarchy of the heterogeneous cell populations in a tumor, and are named CSCs for their similar hierarchical position and properties compared to classic stem cells in development. CSCs also share similar gene expression patterns with normal stem cells (Pece et al., 2010; Shipitsin et al., 2007; Ben-Porath et al., 2008; Shimono et al., 2009), including expression of pluripotency-associated genes and loss of expression of lineage-specific genes concomitant with aberrant expression of genes normally restricted to other cell types. This confers CSCs the ability to derive a phenotypically diverse population of cells that can recapitulate the heterogeneity of a tumor, as well as the plasticity to mimic other cell types and adapt to microenvironmental changes. In addition CSCs phenocopy their normal counterparts by expressing high levels of ATP-binding cassette (ABC) drug transporters and remaining dormant in their established tissue (tumor), leading to refractoriness to chemotherapy and radiotherapy that target actively proliferating cancer cells [reviewed in (Visvader and Lindeman, 2008; Ailles and Weissman, 2007)].

CSCs have been characterized from solid tumors of the breast, brain, prostate, colon, pancreas, head, neck, and lung (Al-Hajj et al., 2003; Singh et al., 2004; O'Brien et al., 2007; Ricci-Vitiani et al., 2007; Patrawala et al., 2006; Li et al., 2007; Prince et al., 2007; Eramo et al., 2008). In most of these studies, CSCs are prospectively isolated by immunosorting based on the expression of various stemness or multi-lineage related surface markers. The molecular markers expressed by CSCs, however, vary depending on the origin and the classification of the cancer. Those used to purify breast cancer stem cells (BCSCs) include CD44 $^{+}$/CD24$^{-/low}$, aldehyde dehydrogenase 1 (ALDH1), CD133/prominin-1, epithelial specific antigen (ESA), mucin 1 and CD49f/integrin α6, etc. (Charafe-Jauffret et al., 2009; Dontu, 2008). However, the BCSC populations enriched using these different surface markers barely overlap, and only a small fraction of the purified cells are tumorigenic in transplanted mice. A functional enrichment strategy relying on the characteristics of stem/progenitor cells to escape anoikis and grow into spheres in anchorage-independent conditions has been exploited from the culturing of neural stem cells, and has been successfully used to obtain highly enriched and functional mammary stem/progenitor cells from both normal and cancerous breast tissue as well as from breast cancer cell lines (Dontu et al., 2003). The sphere-forming efficiency assay is thereby used to assess the number of stem/progenitor cells in the bulk of normal or cancer cells, and ranges from 0.1% to 0.7% in normal mammary epithelial cells and 1–3% in breast cancer cell lines (Charafe-Jauffret et al., 2009; Dontu et al., 2003). Genes that are differentially

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expressed by sphere cells highlight pathways implicated in maintaining the stem cell status, indicating that sphere formation is a good measure of stemness to some extent. Another strategy to enrich for normal and malignant mammary stem cells is based on the activity of ALDH1, a stem cell marker and detoxifying enzyme regulating metabolism of retinal to retinoic acid in stem cell differentiation (Chute et al., 2006). Using a fluorescent ALDEFLUOR assay, Ginestier et al. demonstrated that high ALDH1 activity identifies the CSC population capable of self-renewing and repopulating a heterogeneous tumor. Expression of ALDH1 in primary breast tumors correlates with poor prognosis in patients (Ginestier et al., 2007). ALDEFLUOR-positive populations also exists in breast cancer cell lines and display stem cell properties, including high tumorigenicity and a gene expression profile highlighting genes involved in stem cell function (Charafe-Jauffret et al., 2009).

The self-renewal and differentiation of CSCs are simultaneously regulated by intrinsic (cancer cell-endowed) and extrinsic (microenvironmental) factors. Several pathways have been shown to play roles in the regulation of BCSCs, including Notch, Hedgehog, Wnt/β-catenin, p53 and the TGF-β family (Shipitsin et al., 2007; Liu et al., 2009; Cicalese et al., 2009; Lobo et al., 2007; Watabe and Miyazono, 2009; Dontu et al., 2004). In a solid tumor, CSCs and their progeny are located in a specialized niche provided by the surrounding more differentiated tumor cells and stromal cells, as well as the unique extracellular matrix in the tumor microenvironment. The niche provides both mechanical and chemical stimuli to the CSCs to facilitate their proliferation and survival. As reviewed in the following sections, paracrine signals from the niche cells have shown a critical role in controlling the fate of CSCs.

2. Cytokines regulating the fate of CSCs

2.1. IL-6

In the exploration of the origins of CSCs and their relationships to non-stem cancer cells (NSCCs), a critical role for interleukin-6 (IL-6) in controlling the dynamic equilibrium between CSCs and NSCCs has been identified. Using an inducible breast tumor model, Iliopoulos et al. showed that the $CD44^{high}/CD24^{low}$ CSC population is generated directly from non-transformed MCF10A cells during early oncogenic transformation by a Src derivative. These CSCs are endowed with the typical stem-like properties of being highly spheregenic, tumorigenic, and resistant to chemotherapeutic agents. When cultured separately, purified CSCs rapidly differentiate into NSCCs, eventually maintaining a minor population of ~10%, whereas purified NSCCs rarely convert to CSCs *in vitro*, suggesting an unstable state for CSCs. However, in a mixed population, NSCCs can be converted to CSCs both *in vitro* and *in vivo* in response to exogenous or CSC-secreted IL-6 (Wang et al., 2011). This study indicates that in some solid tumors, such as breast and prostate, CSCs do not arise from normal stem cells in the tissue, and, unlike normal stem cells that are at the top of the cell hierarchy, the conversion between CSCs and NSCCs is bidirectional establishing equilibrium between the two populations. The conversion of NSCCs to CSCs maybe mediated by the induction of an epithelial–mesenchymal transition (EMT), as trastuzumab-induced CSC expansion is primarily mediated by IL-6 and is concomitant with an increase in the expression of Vimentin and Twist and a loss of Ecadherin, EpCAM, and Claudin3 (Korkaya et al., 2012). As different cancer cell lines and primary tumor cells may respond differently to IL-6 as a result of the varying expression levels of IL-6 and its receptor, the NSCC-to-CSC conversion rate and the final proportion of CSCs in a stabilized equilibrium may vary accordingly. In addition, other cytokines that control the differentiation of CSCs into NSCCs will also contribute to the interconversion between the two populations, however IL-6 is able to induce the expression of many of these cytokines hinting that IL-6 may be the master regulator of CSC-inducing cytokines (Korkaya et al., 2012). Therefore, manipulation of these critical cytokines driving the dynamic generation and maintenance of CSCs may serve as a potential strategy for differentiation therapy through reprogramming CSCs into a more differentiated NSCC state that can be easily targeted by chemotherapy drugs.

IL-6 is also produced by non-cancer cells in the tumor microenvironment, including mesenchymal stem cells (MSCs) and various types of immune cells. Bone marrow-derived MSCs can be recruited to the sites of breast tumors and increase the metastatic potency of tumor cells (Karnoub et al., 2007). *In vitro* and *in vivo* results suggest that MSCs can expand the CSC population in breast cancer through cytokine loops involving IL-6 and CXCL7 (Liu et al., 2011). A recent study using a loss-of-function screen identified the IL-6/JAK2/Stat3 pathway to be preferentially active in $CD44^{+}CD24^{-}$ breast cancer cells compared with other cancer cell types (Marotta et al., 2011), suggesting that these cells with the CSC properties undergo more robust IL-6 signaling than NSCCs, as a result of increased IL-6 secretion and/or IL-6 responsiveness in CSCs. This increased dependence of IL-6/JAK2/Stat3 pathway in CSCs provides a therapeutic strategy to specifically and effectively target CSCs.

2.2. IL-8

Initial studies on inflammatory cytokine interleukin-8 (IL-8) indicated that it may serve as a prognostic marker for breast cancer as serum levels of IL-8 correlate positively with metastatic breast cancer and enhanced IL-8 expression correlates with ER-negative breast cancer which has a worse prognosis than ER-positive breast cancer (Benoy et al., 2004; Yao et al., 2007). However, recent studies have shown that the level of IL-8 in the tumor itself is not a good prognostic indicator when comparing tumors of a similar histological grade, indicating that the expression of IL-8 is not as important as its receptor CXCR1 (IL-8RA) (Zuccari et al., 2012). CXCR1 is highly expressed in cancer cells and signalling through IL-8/CXCR1 enhances cell proliferation, migration, and invasion and is correlated with metastatic cancer (Waugh and Wilson, 2008). IL-8 has also been shown to regulate BCSCs as gene expression profiling of the ALDEFLUOR-positive populations from different breast cancer cell lines reveal consistent expression of CXCR1, suggesting a role of IL-8 signaling in CSC function. Recombinant IL-8 increases sphere formation and expansion of the ALDEFLUOR-positive population in breast cancer cell lines through promoting CSC self-renewal while also inducing the chemotaxis of CSCs, which potentially contributes to cancer metastasis (Charafe-Jauffret et al., 2009; Ginestier et al., 2010). Recombinant IL-8 is not able to induce chemotaxis of ALDEFLUOR-negative cells but is able to enhance mammosphere formation of normal breast epithelial cells, hinting that IL-8 may act specifically on both normal breast and breast cancer stem cells to enhance their self renewal (Ginestier et al., 2010). IL-8 can be induced as a result of infiltrating mesenchymal stem cells and chemotherapeutic agents such as docetaxel and trastuzumab, suggesting that chemotherapy may be inducing the expansion of drug-resistant CSCs, leading to the development of a drug-resistant and more invasive cancer in the long-term (Korkaya et al., 2012; Liu et al., 2011; Ginestier et al., 2010).

2.3. CCL2 (MCP-1)

The chemokine (C-C motif) ligand 2 (CCL2), also referred to as the monocyte chemotactic protein-1 (MCP-1), is a potent chemoattractant for monocytes and other immune cells to areas of inflammation (Melgarejo et al., 2009), and has also been implicated in breast

cancer progression and metastasis (Soria and Ben-Baruch, 2008). The level of CCL2 is correlated with accumulation of tumor-associated macrophages in the primary breast cancer, and is a significant indicator of early relapse (Ueno et al., 2000; Saji et al., 2001). In solid tumors CCL2 can be produced by both cancer and stromal cells, including monocytes, fibroblasts, and endothelial cells (Strieter et al., 1989; Lazennec and Richmond, 2010), and its expression in each compartment is dynamically regulated by crosstalk between the tumor and the niche. For example, increased expression of CCL2 is detected in bone marrow MSCs following stimulation by leukemia cells resulting in enhanced cancer-promoting capacity of MSCs (de Vasconcellos et al., 2011). Co-culture with MSCs, in turn, induces CCL2 expression in cancer cells (Molloy et al., 2009).

Our group has recently demonstrated that stroma-derived CCL2 facilitates breast tumor formation through promoting the expansion of the BCSC population (Tsuyada et al., 2012). In this study we focused on the regulation of BCSCs by stromal fibroblasts, an important cellular component of the tumor-hosting niche in many human cancers especially breast cancer. Cancer-associated fibroblasts (CAFs) have been shown to promote cancer progression by modulating multiple components in the cancer niche to facilitate tumor growth and invasion (Kalluri and Zeisberg, 2006). Our study showed that compared to normal fibroblasts, primary CAFs and fibroblasts activated by co-cultured breast cancer cells produce higher levels of CCL2, which stimulates the stem cell-like sphere-forming phenotype in breast cancer cells. We further found that CCL2 does not convert the non-sphere-forming cells to the sphere-forming stem/progenitor cells, but rather induces the self-renewing expansion of existing CSCs. Increased CCL2 expression in activated fibroblasts requires Stat3 activation by diverse cancer-secreted cytokines; this in turn induces Notch1 expression in breast cancer cells constituting a "cancer-stroma-cancer" signalling circuit. Using a xenograft model of paired fibroblasts and primary breast tumor cells isolated from the same patient, we showed that inhibition of CCL2 by neutralizing antibody or RNA interference significantly inhibits tumorigenesis and Notch1 expression. In primary breast tumors higher levels of Notch1 and CCL2 are both associated with poor differentiation (Grade 3 tumors), and a significant linear correlation is observed between CCL2 and Notch1. These findings therefore establish a role of stromal fibroblasts, through secreting CCL2, in the cancer-host communication that prompts CSC-mediated disease progression.

2.4. TGF-β

Accumulating evidence has demonstrated a crucial role of the transforming growth factor (TGF) β, a cytokine whose level is often elevated in the tumor microenvironment and associated with advanced cancer staging, in the regulation of CSC function. Several mechanisms through which TGF-β influences the generation and fate of normal and malignant stem cells have been identified and reviewed elsewhere (Watabe and Miyazono, 2009; Tan et al., 2009). Gene expression profiling suggests that the TGF-β pathway is active in $CD44^+$ breast cancer cells that are enriched for CSCs (Shipitsin et al., 2007). TGF-β can increase the number of transformed cells with CSC properties through inducing EMT (Mani et al., 2008; Asiedu et al., 2011). In a recent study (Wang et al., 2011), we observed that exposure to TGF-β increased the population of sphere-initiating breast cancer cells. This is mediated by the microRNA (miRNA) family miR-181, which is upregulated by TGF-β at the post-transcriptional level. Ataxia telangiectasia mutated (ATM), a well-studied tumor suppressor and a target gene of miR-181, exhibits reduced expression in mammospheres and upon TGF-β treatment. Overexpression of miR-181, or depletion of ATM or its substrate CHK2, is sufficient to induce sphere formation in breast cancer cells. This work elucidates a novel mechanism through which the TGF-β pathway regulates the CSC property by interfering with the tumor suppressor ATM.

Other members of the TGF-β super family have been implicated in the development of various organs and the maintenance of embryonic stem cells (ESC) pluripotency (Watabe and Miyazono, 2009). Among them Nodal, a highly conserved potent morphogen that is absent in normal differentiated cells, is aberrantly overexpressed in aggressive melanoma and breast cancer cells. Inhibition of Nodal signalling reduces cancer cell invasiveness, colony formation and tumorigenicity, and promotes the redifferentiation of aggressive cancer cells (Topczewska et al., 2006). The embryonic microenvironment suppresses the tumorigenic effect of Nodal by producing a high level of Lefty, a divergent member of the TGF-β family that inhibits Nodal signalling, which is absent in the tumor microenvironment (Postovit et al., 2008). Nodal and activin have been reported to maintain pluripotency of human ESCs by controlling the expression of Nanog, a critical transcriptional factor for the "stemness" status, through binding of Smad2/3 to *Nanog* promoter (Vallier et al., 2009). Another TGF-β family member, bone morphogenetic protein (BMP), is necessary for the self-renewal of ESCs through inhibition of mitogen-activated protein kinase (MAPK) and induction of the helix-loop-helix protein Id (inhibitor of differentiation) (Watabe and Miyazono, 2009). Taken together temporally and spatially coordinated signalling of the TGF-β ligands, together with other pathways, form a network that regulates embryonic development and body homeostasis. In cancer it is possible that various TGF-β ligands, through reported and unknown pathways, regulate the acquisition and/or maintenance of the stem cell properties in CSCs, and that correction of a malignant stem cell niche (e.g., by inhibition of the CSC-promoting ligands or restoration of the inhibitory ligand Lefty) may contribute to CSC-targeting therapy.

3. Targeting cytokines in the tumor microenvironment

As summarized in Fig. 1 and evidenced in the studies reviewed above the CSC population is dynamically regulated by a variety of

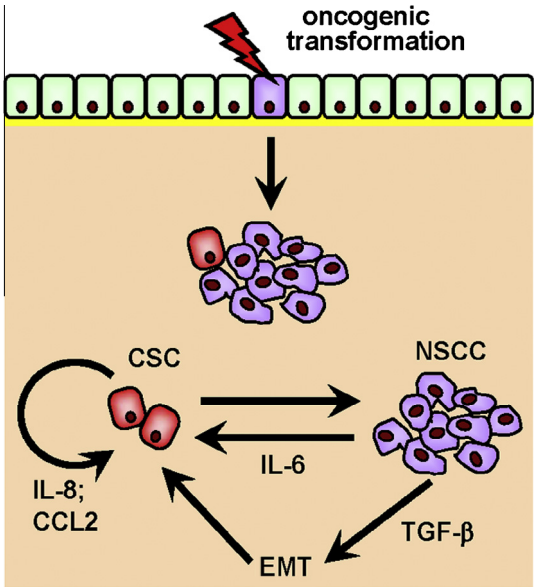

Fig. 1. A schema indicating the reported effects of various cytokines on CSCs. Both CSCs and NSCCs are generated during early oncogenic transformation (Iliopoulos et al., 2011). While CSCs can readily differentiate into NSCCs, NSCCs can also be converted to CSCs in response to IL-6 (Iliopoulos et al., 2011). TGF-β, a potent EMT inducer, may contribute to the generation of CSCs through EMT-mediated mechanism (Mani et al., 2008; Asiedu et al., 2011). In contrast, IL-8 and CCL2 reportedly regulate the self-renewal of CSCs (Ginestier et al., 2010; Tsuyada et al., 2012).

cytokines in the tumor microenvironment during cancer initiation and progression, and both the cancer cells and non-cancer niche cells contribute to the fate determination of CSCs through the secretion of cytokines. As such in different tumors and at different tumor stages, due to the varying types and proportions of infiltrating stromal cells and the varying levels of cytokine production, the major regulator(s) of CSCs may also vary. Therefore, the corresponding cytokine-targeted therapy aiming to suppress the CSC population likely needs to be a personalized treatment. Among the potential targets discussed above, neutralizing antibodies against IL-6 and CCL2 have shown promising efficacy on CSCs *in vitro* and *in vivo* (Iliopoulos et al., 2011; Liu et al., 2011; Tsuyada et al., 2012). The IL-8 receptor CXCR1 has also been shown to be a promising target for therapy as inhibition of CXCR1 with repertaxin or anti-CXCR1 antibodies decreases *in vitro* sphere formation and reduces tumor growth, metastasis, and the ALDEFLUOR-positive population *in vivo*(Ginestier et al., 2010). Repertaxin and anti-CXCR1 antibody treated cells are found to undergo apoptosis after 3 days, suggesting that IL-8 may be important for CSC viability and targeting CXCR1 may be a valuable strategy for targeting breast cancer stemness.

Compared to therapeutic antibodies, which need to be humanized to be applied in patients and often exhibit limited tissue accessibility, small-molecule inhibitors of the critical effectors mediating cytokines' CSC-promoting effect are perhaps a superior group of therapeutic agents. One potential small-molecule inhibitor target is JAK2, a kinase downstream of IL-6 that phosphorylates Stat3. Using a JAK2 inhibitor, Marotta et al. show that inhibition of JAK2 activity significantly reduces the level of phosphorylated Stat3 in $CD44^{+}CD24^{-}$ breast cancer cells, decreases their number, and suppresses the growth of xenograft tumors (Marotta et al., 2011). Inhibition of the Stat3 pathway may be the most efficient way to simultaneously target multiple CSC-promoting cytokines, as Stat3 has been found to induce the expression of all four cytokines discussed herein (i.e., IL-6, IL-8, CCL2 and TGF-β) (Tsuyada et al., 2012; de la Iglesia et al., 2008; Yu et al., 2007). Due to its well demonstrated function in both cancer cells and tumor-infiltrated stromal cells, Stat3 has been long-established as an important therapeutic target in multiple human cancers (Yu et al., 2007). An orally bioavailable dimerization disruptor of Stat3 has been recently developed. This small-molecule inhibitor efficiently inhibits Stat3 phosphorylation and DNA binding, and suppresses the Stat3-mediated malignancy in breast cancer xenografts (Zhang et al., 2012). Whether these effects are accompanied or mediated by a reduction of the CSC population awaits further investigations.

Other potential CSC-targeting strategies have arisen from recent studies. Notch activation has been shown to promote the self-renewal of normal and malignant breast stem cells (Dontu et al., 2004). An important role for Notch signalling in human cancers has been long established (Ranganathan et al., 2011), and several γ-secretase inhibitors (GSIs) are currently in early clinical development as potential Notch-targeting therapeutics. Due to the lack of drugable target in triple-negative breast cancer and the CSC-like characteristics of the cancer subtype, GSI therapy is expected to be an effective treatment for this fraction of breast cancer (Takebe et al., 2011). Since the CSC-promoting effect of CCL2 requires the induction of Notch1 (Tsuyada et al., 2012), Notch-targeting agents may also block the stimulatory effect of stromal fibroblasts on CSCs.

In summary, the recent findings reviewed herein further emphasize the importance of simultaneously targeting cancer cells and the CSC-promoting cytokines in the tumor microenvironment. Future anti-cancer therapies are expected to incorporate the latest stem cell research. As cancer is a progressive disease involving co-evolution of tumor cells and the hosting niche, strategies simultaneously targeting multiple CSC-promoting mechanisms that may feature in different tumor stages may be more effective, especially when combined with the conventional therapies targeting the bulk of the cancer cells.

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